Apoptosis Signal-Regulating Kinase 1 Attenuates Atrial Natriuretic Peptide Secretion†

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ABSTRACT: Atrial natriuretic peptide (ANP) is an endogenous peptide hormone that is synthesized and secreted by the myocardium in health and disease. Although the bioactivity of this molecule has been studied extensively, cellular mechanisms governing its processing and secretion are not fully understood. Through a yeast two-hybrid screen of a cDNA library made from tissue of a failing human heart, we have discovered that the precursor of ANP, natriuretic peptide precursor (NPPA), physically interacts with the N-terminus of apoptosis signal-regulating kinase 1 (ASK1), a kinase believed to be involved in the pathogenesis of heart failure. We demonstrated that NPPA is a substrate of ASK1 in an in vitro kinase assay. Indirect immunofluorescence microscopy shows that, when expressed in Hela cells, ASK1 and NPPA exhibit distinct, but overlapping, staining patterns, suggesting partial colocalization in cells. Additionally, coexpressing wild-type ASK1 with NPPA in Hela cells led to reduced levels of NPPA in the culture medium, suggesting that ASK1 negatively impacts NPPA processing and/or secretion. This negative effect was less pronounced when a dominant-negative allele of ASK1 with deficient kinase activity was coexpressed with NPPA. Because both ASK1 and ANP are associated with pathologic cardiac hypertrophy, their interaction may have pathophysiological and therapeutic relevance.

Atrial natriuretic peptide (ANP)[1] is a peptide hormone of 28 amino acids. It is a member of the natriuretic peptide family that contributes to the regulation of diuresis, natriuresis, and the secretion of other hormones such as aldosterone, vasopressin, and renin (*1*−*6*). Increased plasma levels of ANP are associated with diseases such as heart failure and hypertension, with recent studies supporting a role for ANP as a paracrine regulator of cardiac structure and function in these conditions (*7, 8*). In both healthy and diseased individuals, ANP exerts its effects through cell surface receptors, natriuretic peptide receptor A (NPR-A), natriuretic peptide receptor B (NPR-B), and natriuretic peptide receptor C (NPR-C). NPR-A and NPR-B both have guanylate cyclase activity and act to increase cellular cyclic GMP (cGMP) levels upon activation, while NPR-C has a minimal intracellular domain and serves as a so-called clearance receptor that removes ANP from the circulation (*2*).

Compared with the extensive knowledge of ANP actions and signaling, our understanding of factors regulating ANP synthesis, processing, and secretion is relatively incomplete. In atrial myocardium, ANP is initially synthesized as a prepropeptide of 150 residues. After the prepro form enters the endoplasmic reticulum (ER), its leader sequence is trimmed off, and natriuretic peptide precursor A (NPPA) of 13 kDa is formed and stored in specialized secretory granules. Upon distension of the atrial myocardium, NPPA is released from the granules and cleaved on the plasma membrane by Corin into an N-terminal proANP and the bioactive mature 28-amino acid cyclic peptide (*2, 9*). During pathological hypertrophy and heart failure, NPPA and ANP are also expressed and secreted constitutively from ventricular myocardium by a mechanism different from the regulated, stretch-coupled secretion characteristic of the atrial myocardium (*10, 11*). However, the molecular signals and mechanisms governing ANP processing and secretion, such as factors that affect NPPA folding in ER and its transportation through Golgi to a vesicle during the secretion, are not well-understood.

Apoptosis Signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase kinase kinase, MAP3K5. It shares more than 40% homology at the amino acid level with members of the MAPK family (*12, 13*). Activation of ASK1 was reported under a variety of conditions, such as oxidative stress, ER stress, stimulation by lipopolysaccharide (LPS), cytokines, Fas ligand, G-protein-coupled receptor (GPCR) agonists, and calcium overload (*14, 15*). In turn, ASK1

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[1] Abbreviations: ASK1, apoptosis signal-regulating kinase 1; MAP3K5, mitogen-activated protein kinase kinase kinase 5; MAPK, mitogen-activated protein kinase; NPPA, natriuretic peptide precursor A; ANP, atrial natriuretic peptide; NPR-A, natriuretic peptide precursor A; NPR-B, natriuretic peptide precursor B; NPR-C, natriuretic peptide precursor C; GST, glutathione *S*-transferase; LPS, lipopolysaccharide; GPCR, G-protein-coupled receptor; cGMP, cyclic guanosine monophosphate; GDB domain, Gal DNA binding domain; ROS, reactive oxygen species; ER, endoplasmic reticulum; DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; IgG, immunoglobulin class G; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; WT, wild type; MI, myocardial infarction; TAC, thoracic aortic constriction.

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activation initiates signaling in both JNK and p38 pathways (*16*). The role of ASK1 in mediating cardiac pathology has been under extensive study, with recent investigations indicating that ASK1 serves as a mediator of cytokine and reactive oxygen species (ROS) signaling during the development of cardiomyocyte hypertrophy (*17, 18*).

In this work, we show that N-terminal ASK1 interacts with NPPA expressed in the left ventricle of a hypertrophic human heart in a yeast two-hybrid system. Subsequent in vitro studies demonstrated that NPPA is a substrate of ASK1. When coexpressed with NPPA in Hela cells, wild-type ASK1 negatively impacted the secretion of NPPA into the culture media. Given the role played by ANP in heart failure, this novel interaction suggests a possible mechanism by which ASK1 affects the pathogenesis of heart failure.

MATERIALS AND METHODS

Chemicals and Reagents. Chemicals used in this study were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Restriction enzymes and DNA ligase were from New England Biolabs, Inc. (Beverly, MA). PCRs were carried out using GoTaq Green Master Mix (Promega, Madison, WI). Recombinant ASK1 was purchased from Invitrogen (Carlsbad, CA). DNA isolation kits were from Qiagen (Valencia, CA). Polyclonal antibody against ASK1 was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PDI and anti-Golgin 97 were from Invitrogen. Monoclonal anti-ASK1 was from AbD Serotec (Oxford, U.K.), and polyclonal anti-ANP was from Chemicon (Temecula, CA). Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Plasmid Constructions. (i) pGBKT7-ASK1N2. An HincII−XmnI cDNA fragment encoding the N-terminus of human ASK1 (bases 699−2238) was ligated to the SmaI site of pGBKT7 (Clontech Laboratories, Inc.), in frame to the yeast GAL4-DNA binding (GDB) domain at the 5′ end. This plasmid allows the constitutive expression of a fusion protein of the GDB domain and the N-terminus of ASK1 in yeast.

(ii) pGST/NPPA. A cDNA fragment containing the coding region of NPPA immediately after the leader sequence was amplified by PCR using primers 5′-tgaatcccatgtacaatgccg (forward) and 5′-gagcagcgccctcagcttgc (reverse). A two-hybrid library plasmid containing cDNA of NPPA, retrieved from a positive colony of the screen, was used as the template. The amplified cDNA was cloned into the pGEMT Easy PCR cloning vector (Promega). An EcoRI fragment was excised and ligated into pGEX4T-1 (Amersham Biosciences, Pittsburgh, PA) at an EcoRI site. The clone was sequenced to verify that no mutations were introduced during the PCR amplification. This plasmid expresses a fusion protein of glutathione *S*-transferase (GST) and NPPA in *Escherichia coli*.

(iii) pCMV-NPPA and pCMV-ctrl. The entire coding region, including the leader sequence of NPPA cDNA, was PCR-amplified using primers 5′-gcatgagctccttctccacc (forward) and 5′-gatcaagcgtaatctggtacgtcgtatgggtactcgtaccggaagctgttacagc (reverse) from the same two-hybrid library plasmid described above. The PCR product was cloned into pGEMT Easy. A NotI fragment from this plasmid was subsequently ligated with the NotI-linearized vector of the pCMV-SPORT-β-Gal (Invitrogen). The linearized pCMV-SPORT-β-Gal vector was also self-relegated to form pCMV-ctrl. The pCMV-NPPA plasmid expresses NPPA, including the leader sequence, when transfected into a Hela cell line, whereas pCMV-ctrl serves as a vector control. The pCMV-NPPA plasmid was sequenced to confirm that no mutation was introduced.

(iv) pCMV-ASK1. The full-length cDNA of ASK1 was amplified by PCR from a TrueClone plasmid carrying human ASK1 cDNA (OriGene Technologies, Inc., Rockville, MD) using primers 5′-ccatgagcacggaggcggacga (forward) and 5′-tctagatcaagtctgtttgtttcgaaa (reverse). The PCR product was cloned into pCMVScript (Stratagene, La Jolla, CA) at the SrfI site. A NotI−ApaI fragment was excised from this plasmid and ligated with NotI−ApaI-linearized pCMVSPORT-β-GAL (Invitrogen) vector. The wild-type sequence of ASK1 in this plasmid was confirmed by sequencing.

(v) pCMV-K709R. A QuikChange XL site-directed mutagenesis kit (Stratagene) was used to introduce a point mutation that converts a lysine at amino acid 709 to an arginine in ASK1 on the pCMV-ASK1 plasmid. The resulting pCMV-K709R plasmid expresses the mutant allele of ASK1 that possesses reduced, though not absent, kinase activity.

Yeast Two-Hybrid Screen. A BD Matchmaker Library Construction and Screening Kit (Clontech Laboratories, Inc.) was used to make a cDNA library of left ventricle tissue from an explanted failing human heart, according to the manufacturer's protocol. Plasmid pGBKT7-ASK1N2 was introduced into strain AH109. The resulting transformants were purified on plate by selecting for single colonies that display the appropriate markers. AH109 carrying pGBKT7-ASK1N2 was subsequently used as the host strain for cDNA library transformation. Positive clones that are able to grow on synthetic minimal medium lacking tryptophan, histidine, and leucine were selected. They were further confirmed by measuring the β-galactosidase activity, according to the manufacturer's instruction. Library plasmids carrying cDNA inserts were retrieved with a yeast plasmid isolation kit (Clontech) and amplified in *E. coli* for further analysis.

Protein Purifications. The expression of the GST−NPPA fusion protein from the expression vector, pGST-NPPA, in *E. coli* strain BL21(DE3) pLys (Stratagene) was induced by IPTG, at a final concentration of 0.1 mM for 3 h. The expressed fusion protein was purified using a glutathione Sepharose 4B column (Amersham) following the manufacturer's instructions. Purified fusion protein was desalted using Zeba Desalt Spin Columns (Pierce, Rockford, IL). As a control, GST was expressed from vector pGEX4T-1 and purified using the same method.

Kinase Assay. In the assay reaction, 1 μg of purified GST−NPPA fusion protein and 0.5 μg of recombinant ASK1 were used. The reaction buffer contained 50 mM Tris (pH 7.5), 15 mM $MgCl_2$, 0.1 mM EGTA, 2.5 mM DTT, 1 mM ATP, Halt phosphotase inhibitor (Pierce), and protease inhibitor (Roche Applied Science, Indianapolis, IN). The reaction mixture was incubated at 30 °C for 40 min, and the reaction was terminated by adding Lammeli buffer and heating at 100 °C for 5 min. Proteins were separated on a 13% SDS−PAGE. Phosphorylated proteins were detected by staining the gel with Pro-Q Diamond Phosphoprotein Gel Stain (Invitrogen) according to the manufacturer's instructions. The image was visualized using a Typhoon 9410

Variable Mode Imager (Amersham). The gel was subsequently stained with SYPRO Ruby (Invitrogen), and the image was scanned again for total protein visualization.

Cell Culture and Transfection. Hela cells (American type Culture Collection, Manassas, VA) were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA) at 37 °C in 5% CO_2. The cells were grown to 60−80% confluence in a 12-well plate and transfected with a TransIt-HelaMONSTER transfection kit (Mirus, Madison, WI) following the manufacturer's instructions. For each transfection, 0.5 μg of DNA and 1.5 μL of TransIt reagent were used. Transfected cells were cultured in the same medium for 48 h, and both the cells and the medium were harvested for analysis.

Indirect Immunofluorescence Microscopy. Cells were fixed with 4% formaldehyde for 15 min, followed by permeabilization with 0.5% Triton X-100. Permeabilized cells were blocked with 1% normal goat serum. The cells were then incubated with primary antibodies at 4 °C overnight, washed with PBS, and incubated with FITC- and/or TRITC-conjugated secondary antibodies for 2 h. After incubation, cells were mounted on a glass slide using mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) and visualized on a Nikon Eclipse 80i fluorescence microscope (Nikon Corp.) equipped with a SPOT RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Immunoblot Analysis and β-Galactosidase Assay. Immunoblot analyses were carried out according to a standard protocol (*19*). Cells were washed with ice-cold PBS and total cellular proteins isolated on plate using 40 μL (per well) of M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, Inc.) containing Complete Mini, a cocktail of protease inhibitors (Roche Applied Science). The lysates were cleared of debris by centrifugation. Proteins in the cell lysates were separated via SDS−PAGE and transferred to a PVDF membrane (Millipore). Primary antibodies used were polyclonal anti-ASK1 (Santa Cruz Biotechnology), monoclonal anti-ANP (AbD Serotec), and polyclonal anti-β-actin (Cell Signaling Technology, Beverly, MA). Secondary antibodies used were HRP-conjugated goat anti-rabbit IgG (Amersham) and HRP-conjugated goat anti-mouse IgG (Amersham). Chemiluminescence reagents (Amersham) were used to detect the antigen−antibody immune complex on the membrane. For the β-galactosidase assay, 5 μL of the lysate was diluted with 30 μL of M-PER, and 35 μL of All-in-One reagent from the Mammalian β-Galactosidase Assay kit (Pierce Biotechnology, Inc.) was added. The reaction mix was incubated at 37 °C for 30 min. The absorbance at 405 nm was measured on an AD 200 plate reader (Beckman Coulter).

Enzyme-Linked Immunosorbent Assay (ELISA). A 96-well high-binding microplate (Greiner Bio-one) was coated with a capture antibody, a polyclonal goat antibody against the C-terminus of human NPPA (Santa Cruz), at 16 μg/mL in PBS overnight at 4 °C. Nonspecific binding sites were blocked with PBS containing 1% bovine serum albumin. ANP standards and culture medium samples were loaded into the wells and allowed to incubate for 2 h. Detection of ANP was done by incubating the plate for 1 h with a mouse monoclonal antibody against ANP (AbD Serotec, diluted 1:1000 in the blocking buffer), followed by incubation for 1 h with biotinylated goat anti-mouse antibody (R&D Systems) diluted 1:2500 in the blocking buffer and incubation for 30 min with straptavidin−horseradish peroxidase (HRP) conjugates (R&D Systems). The plate was washed four times with PBS containing 0.05% Tween 20 between incubations. After a final wash, the plate was developed with TMB and stopped with 2 N HCl. The absorbance was measured at 450 nm in AD 200 plate reader (Beckman Coulter). Results from multiple experiments were averaged, and comparisons between experimental conditions were analyzed using a Student's *t* test.

RESULTS

Natriuretic Peptide Precursor A (NPPA) Interacts with the N-Terminus of ASK1 in a Yeast Two-Hybrid System. A cDNA library was generated using RNA isolated from left ventricular tissue of an explanted failing human heart (see Materials and Methods). To identify interaction partners of ASK1 in this library, we constructed a plasmid that expresses a bait, N-terminal ASK1 (residues 225−746), fused to the GAL4 DNA binding domain of yeast *Saccharomyces cerevisiae* after transformation into yeast cells (Figure 1A). The fusion protein was placed under the control of the yeast ADH1 promoter in plasmid pGBK-T7-ASK1N2. Both the bait construct and the cDNA library were transformed into yeast strain AH109, a tester strain (Materials and Methods). Positive colonies, expressing potential ASK1-interacting proteins from the library, were isolated as those capable of growing on synthetic medium lacking Trp, His, and Leu (Materials and Methods). Eight hundred such colonies were obtained from an estimated 250000 transformants. Sequencing analysis of the cDNA inserts from positive clones showed that a vast majority of the inserts containing cDNA encoding either cardiac troponin I (cTnI) (57%) or natriuretic peptide precursor A (NPPA) (35%). Since the interaction of ASK1 and cTnI has been previously reported (*20*), we focused on the interaction between ASK1 and NPPA in this study.

The interaction between the N-terminus of ASK1 and NPPA was confirmed by cotransformation of the tester strain with the recovered prey plasmid containing NPPA cDNA and either the bait plasmid, pGBK-T7-ASK1N2, or the vector plasmid, pGBK-T7, without the ASK1 moiety. As expected, cells carrying pGBK-T7-ASK1N2 and the prey plasmid were able to grow on synthetic minimal medium lacking His, Trp, and Leu (Figure 1B). In contrast, cells carrying pGBK-T7 without the ASK1 moiety and the prey plasmids cannot grow on the same medium (Figure 1C). The results indicated that the N-terminus of ASK1 is required for the activation of reporter gene expression. By bringing together the DNA binding domain and the transcription activation domain of GAL4 through the interaction of ASK1 and NPPA, cells were able to express the HIS3 reporter gene that supports the growth on medium lacking histidine. We also tested the C-terminus of ASK1 for its ability to interact with NPPA, and the result was negative (data not shown). Thus, the interaction between ASK1 and NPPA observed with the two-hybrid screen was specific and limited to the N-terminal domain of ASK1.

NPPA Is Phosphorylated in Vitro by ASK1. The interaction of NPPA and ASK1N2 led us to test the possibility of NPPA being a substrate for ASK1. We constructed a plasmid, pGST-NPPA, expressing a fusion protein of GST and NPPA

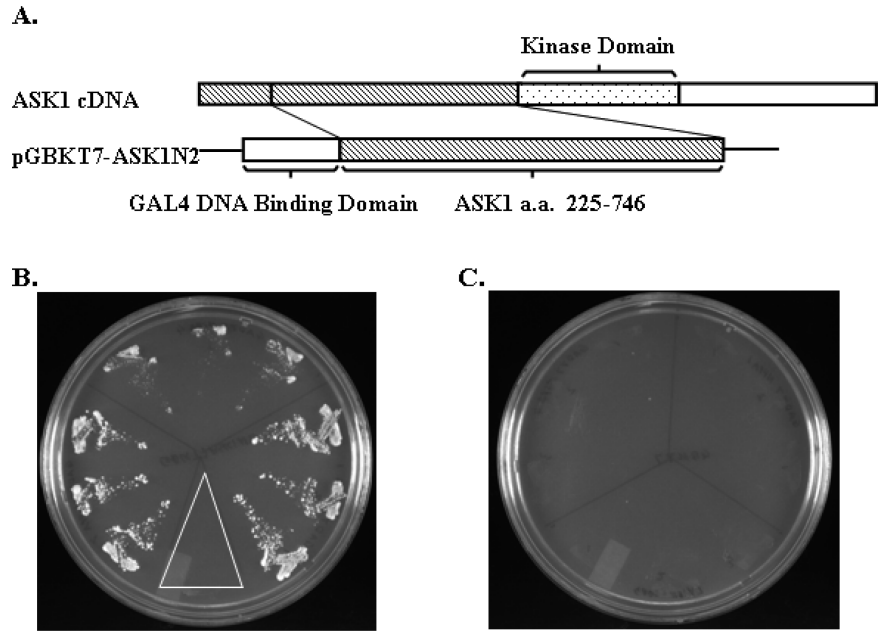

FIGURE 1: Growth of a yeast two-hybrid reporter strain is dependent on the interaction of NPPA and N-ASK1. (A) Diagram depicting the gene structure of the bait plasmid containing the fusion protein. (B) Cells coexpressing GAL4 activation domain−NPPA and GAL4 DNA binding domain−ASK1N2 species are viable on medium lacking histidine. Cells expressing only the GAL4 DNA binding domain−ASK1N2 species are not viable (triangle region). (C) Cells coexpressing the GAL4 activation domain−NPPA species and the GAL4 DNA binding domain are not viable on the same medium.

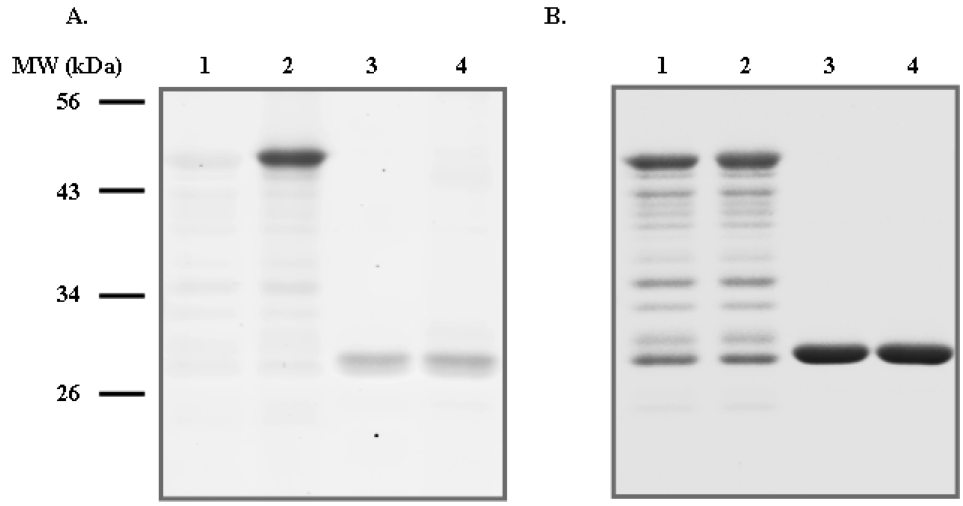

FIGURE 2: NPPA is phosphorylated by ASK1. (A) Pro-Q Diamond staining. NPPA is not phosphorylated when incubated with GST (lane 1). NPPA is phosphorylated when incubated with ASK1 (lane 2). GST is not autophosphorylated (lane 3) or phosphorylated by ASK1 (lane 4). (B) SYPRO Ruby staining. The same gel previously stained with Pro-Q Diamond was stained with SYPRO Ruby to show equal loading of all the samples.

from the IPTG inducible Tac promoter. After transformation of *E. coli* strain BL21(DE2)pLys with pGST-NPPA, the fusion protein was affinity-purified using a glutathione 4B column (Materials and Methods) and subsequently used in a kinase reaction as a substrate for ASK1. GST alone was also purified and used as a control in the kinase reactions. Phosphorylation of NPPA by ASK1 was detected by electrophoresis of the kinase reaction products followed by Pro-Q Diamond and SYPRO Ruby staining of the gel (Materials and Methods).

As shown in Figure 2, the GST−NPPA fusion protein was phosphorylated by ASK1 (lane 2), which is evident from the strong staining of the fusion protein. When ASK1 was replaced with GST, no phosphorylation of NPPA was observed (Figure 2A, lane 1), indicating that the observed phosphorylation was dependent on the presence of ASK1, rather than any contaminating bacterial kinase activity in the purified GST−NPPA protein preparation. Similarly, when GST was used to replace the GST−NPPA fusion protein, we observed a background staining of GST similar to the staining pattern of GST alone without ASK1 in the reaction (Figure 2A, lanes 3 and 4). This suggests that GST is not phosphorylated by ASK1. Thus, the observed phosphorylation of the GST−NPPA fusion protein by ASK1 must have occurred on the NPPA moiety. SYPRO Ruby staining of the gel confirmed equal loading of the lanes (Figure 2B). Taken together, our results showed that ASK1 specifically phosphorylates the NPPA moiety of the GST−NPPA fusion protein in vitro. Combined with the fact that ASK1 and NPPA interact with each other inside yeast cells, it strongly suggests that NPPA could be a substrate of ASK1 in vivo, under certain conditions.

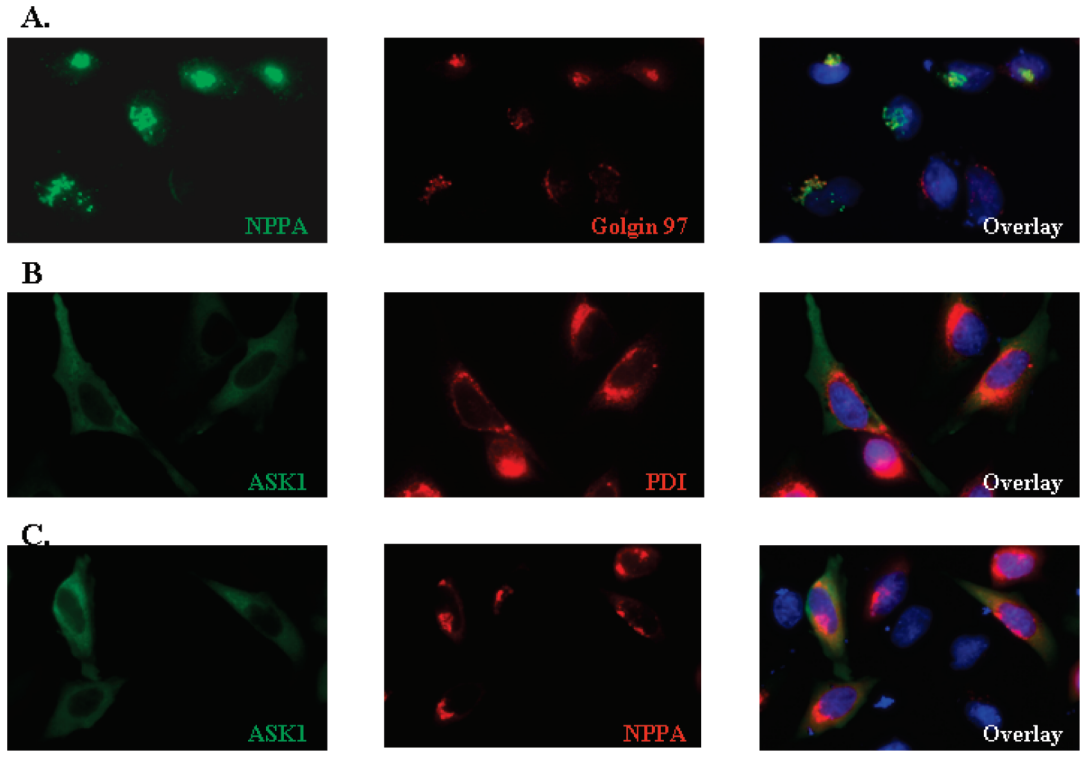

FIGURE 3: Subcellular localization of ASK and NPPA. (A) Hela cells transfected with pCMV-NPPA. The cells were doubly labeled with antibodies against ANP and Golgin 97, a marker for the Golgi apparatus. (B) Hela cells transfected with pCMV-ASK1 and doubly labeled with antibodies against ASK1 and PDI, an ER marker protein. (C) ASK1 and NPPA are coexpressed in Hela cells. The cells were doubly labeled with antibodies against ASK1 and ANP. Cells were fixed 48 h post-transfection and fixed in 4% formaldehyde before antibody incubations.

NPPA and ASK1 Exhibit Overlapping Intracellular Localization in Hela Cells. We used indirect immunofluorescence microscopy to examine the subcellular localization of NPPA and ASK1. Hela cells were transfected with an NPPA-expressing plasmid, pCMV-NPPA, and fixed 48 h post-transfection. The intracellular location of NPPA was determined by using a primary polyclonal antibody against NPPA and a FITC-conjugated secondary antibody, and the location of Golgi was revealed by the staining of a primary monoclonal antibody against Golgin 97, a maker protein for the Golgi, and a different TRITC-conjugated secondary antibody (see Materials and Methods). The staining patterns showed that the majority of NPPA colocalized with Golgin 97 (Figure 3A), indicating that NPPA expressed from the plasmid was properly targeted to the secretion machinery and entered the pathway for ultimate exocytosis.

Similarly, Hela cells were transfected with ASK1 expression plasmid pCMV-ASK1. The cells were fixed and doubly labeled with anti-ASK1 and anti-protein disulfide isomerase (PDI) antibody, an ER marker protein. As shown in Figure 3B, ASK1 assumes a general cytoplasmic staining pattern but with a stronger presence near the nuclear envelope and the ER. The staining patterns of ASK1 and NPPA overlapped in the region of the ER (Figure 3C). The localization data suggest that ASK1 may come into contact with NPPA in the ER or on the ER membrane, before the protein enters the first organelle in the secretion pathway. Finally, the level of expression of NPPA was not detectably altered by the expression of ASK1, based on double labeling results in cells coexpressing both NPPA and ASK1 (Figure 3C). This was also confirmed by immunoblot analysis (Figure 4B). In addition to pCMV-ASK1, we also transfected pCMV-K709R

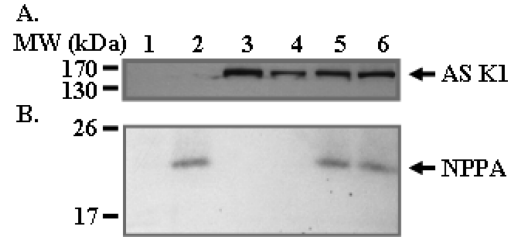

FIGURE 4: Expression of NPPA in Hela cells cotransfected to also express WT ASK1 and the K709R mutant form of ASK1. Hela cell lysates made from untransfected (lane 1), pCMV-NPPA/pCMV-ctrl-transfected (lane 2), pCMV-ASK1/pCMV-ctrl-transfected (lane 3), pCMV-K709R/pCMV-ctrl-transfected (lane 4), pCMV-ASK1/pCMV-NPPA-transfected (lane 5), and pCMV-K709R/pCMV-NPPA-transfected (lane 6) cells were separated via SDS−PAGE and transferred to a membrane followed by probing with a polyclonal anti-ASK1 antibody (A) and monoclonal anti-ANP antibody (B). No significant changes in cellular NPPA levels were observed when the cells were cotransfected with expression plasmids of ASK1 or ASK1 K709R.

that expressed a mutant allele of ASK1 with lysine 709 replaced with arginine. This point mutation was reported to reduce the kinase activity of the protein (*21*).

NPPA Secretion Is Reduced by Coexpression with ASK1. Secretion of NPPA into the circulation is a critical step in the fulfillment of its biological function. To assess the effect of ASK1 on NPPA secretion, we measured the NPPA levels in culture medium of Hela cells expressing both NPPA and ASK1. Cells were seeded at the same inoculum and transfected in triplicate with combinations of plasmids: pCMV-NPPA and a vector only plasmid, pCMV-ctrl; pCMV-NPPA and pCMV-ASK1; pCMV-NPPA and pCMV-K709R (with the total amount of DNA kept constant). As a

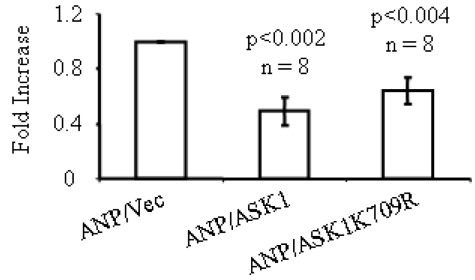

FIGURE 5: Secretion of NPPA from transfected Hela cells. Hela cells were transfected with combinations of plasmids pCMV-NPPA, empty vector (pCMV-ctrl), pCMV-ASK1, or pCMV-K709R. The amount of NPPA secreted into the culture medium was measured by an ELISA and normalized to the β-galactosidase activity of each transfection. The ratios of normalized NPPA levels were graphed. Cotransfection of NPPA and ASK1 led to an ~50% reduction in the level of NPPA in the medium. Error bars denote standard errors.

control for transfection efficiency, DNA of pCMV-SPORT-β-Gal (Invitrogen) that expresses Lac-Z was included in each transfection. Culture medium was collected 48 h post-transfection, and debris was removed by centrifugation. The cells were lysed on plate, and the expression of NPPA and ASK1 were confirmed by immunoblot analysis (Figure 4). The result shows that both proteins were expressed after the transfection of the plasmids. NPPA levels in these samples were measured using an ELISA and adjusted for variation of transfection efficiencies by β-galactosidase activity in the cell lysates. The ratios of NPPA in the culture media of cells expressing the combinations of plasmids were determined. As shown in Figure 5, coexpression of ASK1 with NPPA reduced the concentration of NPPA in the medium compared to coexpression of the vector plasmid with NPPA (50% reduction; $p < 0.002$, $n = 8$). Furthermore, coexpression of a kinase-deficient ASK1, K709R, had a slightly reduced effect (35% reduction; $p < 0.004$, $n = 8$) on the level of NPPA in the medium (Figure 5). However, the comparison between the ANP/ASK1 and ANP/K709 cotransfections reveals no significant difference ($p = 0.15$). These results indicate that ASK1 expression reduces the level of NPPA secretion via an action that may involve ASK1 kinase activity.

DISCUSSION

In this study, we have shown that NPPA interacts with ASK1 in the yeast two-hybrid system. Furthermore, NPPA expressed from *E. coli* is phosphorylated by ASK1 in vitro. When coexpressed, ASK1 and NPPA exhibit overlapping spatial domains in the ER region. In addition, secretion of NPPA is attenuated upon ASK1 expression, and this functional effect may depend upon ASK1 kinase activity. Taken together, our results suggest that ASK1 phosphorylation of NPPA alters the prohormone's intracellular processing and impedes its secretion. These findings point to a potential in vivo interaction between ASK1 and NPPA, particularly in states like hypertrophy and heart failure where the disease has been associated with increased myocardial abundance of both molecules (*22, 23*).

The yeast two-hybrid screen has been used previously in identifying the interacting partners of ASK1 in various tissues (*20, 24*−*28*). For instance, two of these studies used cardiac cDNA libraries; albeit one was of a murine origin. In the screen of the murine library with the calcium binding B subunit of calcineurin (CnB) as bait, ASK1 was identified as an interaction partner, and dephosphorylation of ASK1 at serine 967 by calcineurin led to ASK1 activation (*27*). In the other study, He et al. used ASK1 lacking the noncatalytic N-terminus domain as bait to screen a normal human heart cDNA library and found cardiac troponin I (cTnI) as an interaction partner (*20*). Phosphorylation of troponin I by ASK1 resulted in impaired cardiac myocyte contractility. In this study, we find NPPA, in addition to cTnI, interacted with the N-terminal domain of ASK1. The discovery of this additional interacting protein could be attributed to either the use of a different cDNA library, made from a severely myopathic left ventricle, or the use of a different domain of ASK1 as the bait. The first possibility would suggest that this interaction could be disease-specific, given that NPPA expression may be absent in ventricles from hearts without hypertrophy or failure (*29*). The second possibility would suggest that the interaction could be specific to the N-terminal domain of ASK1. Indeed, in separate studies, we did not observe any interaction between the C-terminal catalytic domain of NPPA and ASK1 (data not shown). The specificity of protein−protein interactions often depends on their noncatalytic domains. It has been shown by several studies that many kinases, including the MAPK kinases, rely on their noncatalytic domains, the docking sites, to achieve specific protein−protein interactions (*30*). Thus, the ASK1−NPPA interaction could be domain- and/or disease-specific.

The association of ASK1 with cardiac disease has been studied extensively. ASK1 activation was reported in p38/JNK-mediated apoptosis and due to ER and oxidative stress induced by pathological stress induced by angiotensin II stimulation and autoxidation of catecholamines in the heart (*15, 31*). Studies using cell culture methodology indicate that ASK1 is involved in cardiac myocyte hypertrophy, apoptosis, and collagen deposition (*32*−*34*). In vivo, wild-type (WT) mice exhibited a >17-fold increase in ASK1 activity without an increase in the ASKI protein level, 2 days after either experimental myocardial infarction (Ml) or thoracic aortic constriction (TAC). Conversely, ASK1-deficient mice ($ASK1^{-/-}$) had attenuated pathologic remodeling following experimental MI or TAC. Interestingly, WT and $ASK^{-/-}$ mice exhibited similar degrees of p38 activation, and only limited reductions in JNK activation, with either MI or TAC (*17*). These studies raise the possibility of MAPK-independent actions of ASK1 activation following pathological stress.

The secretion of mature ANP requires a series of steps. Removing the leader sequence and assuring proper folding of NPPA in the ER are early steps that precede passage through the Golgi apparatus and packaging in a vesicle that ultimately fuses with the plasma membrane. Many factors, such as regulators of protein folding, sorting, modification, and transportation, could affect this process along the way and determine the destination of the protein. The ER serves critical functions in this process by ensuring the proper folding of a secretory protein and removing misfolded proteins for degradation through the ubiqutin pathway in the proteasome, thus reducing the level of accumulation of misfolded protein that could cause ER stress and apoptosis (*35, 36*). Given the fact that ASK1 phosphorylates NPPA in vitro and partially colocalizes with NPPA in the ER, it is possible that

ASK1 could carry out the phosphorylation of NPPA in cells. In fact, it was reported that NPPA was partially phosphorylated in cultured neonatal rat cardiac myocytes, although the biological significance of this was not clear (*37*). Our results suggest that ANP secretion could be affected by the possible removal of the phosphorylated population of NPPA from the ER. By analogy, it has been shown that when a phosphorylation site in H^+-ATPase in yeast *S. cerevisiae* was mutated, the folding of the protein became defective and the normal movement of the protein along the secretory pathway was interrupted (*38*). Consistent with this, we demonstrated that ASK1 K709R, a "kinase deficient" allele that resulted from a point mutation of lysine 709 to arginine, inhibited NPPA secretion to a lesser extent (35%, $p < 0.004$) when compared to WT ASK1 (50%, $p < 0.002$). The kinase deficient allele has previously been shown to retain some residual kinase activity (*21*). When this allele is overexpressed, this residual activity may allow a limited inhibitory effect on the secretion process, although the effect would be weaker than that of the wild-type allele. Conceivably, ASK1 might also alter in vivo ANP secretion by affecting the ability of the enzyme Corin to cleave the prohormone NPPA to the mature 28-amino acid ANP peptide. However, in NPPA-transfected Hela cells, immunoblots revealed no difference in size of the NPPA and ANP protein from intracellular and extracellular sources, consistent with the absence of Corin expression in these cells (data not shown). Thus, further studies utilizing cells expressing Corin will be required to examine whether ASK1 affects NPPA cleavage by Corin.

Recognizing that ANP is critically involved in the regulation of blood pressure, body fluid homeostasis, and cardiac structure, we suggest that the ASK1−NPPA interaction could play a role in the pathogensis of hypertrophy. In cultured cells, ANP demonstrates antihypertrophic (*5, 7*) and antifibrotic (*39*) functions, and mice lacking the A-type natriuretic peptide receptor (NPR-A) developed cardiac hypertrophy and fibrosis independent of their blood pressure (*8*). Moreover, studies of transgenic mice lacking the ANP precursor gene demonstrate an increased susceptibility to heart failure and myocardial fibrosis (*40, 41*). In this context, our findings provide a possible route for ASK1 to exert its influence in promoting the cardiac dysfunction: by negatively regulating the processing and secretion of NPPA, thereby lowering both circulating and myocardial ANP concentrations. Additionally, the ASK1−NPPA interaction could also impact the pathogenesis of cardiac hypertrophy by promoting apoptosis resulting from ER stress due to misfolded NPPA. The ER stress could contribute to p38/JNK activation signaling apoptosis. Thus, the results from this study support the current knowledge regarding the functions of ASK1. It is possible that the mechanism involving ASK1 in the development of cardiac hypertrophy could include both MAPK-dependent and -independent pathways. Though it would be ideal to carry out similar experiments in primary human cardiac myocytes, difficulties in maintaining these cells in culture have impeded our progress to date. Animal models with a reduced level of ASK1 expression or ASK1 kinase activity may offer another alternative for confirming the functional significance of the interaction between ASK1 and myocardial NPPA secretion.

In conclusion, we have identified a novel and functionally significant interaction between ASK1 and NPPA, two molecules that have each been found to be associated with the failing myocardium. Because the biological actions of endogenous ANP are beneficial for maintaining body fluid homeostasis and limiting adverse cardiac remodeling, the downregulation of ANP secretion by ASK1 appears to be unfavorable and likely to promote progression of heart failure and cardiac dysfunction. Such a perspective is consistent with the protective effects of ASK1 ablation in transgenic mice exposed to pathological manipulations. Our findings thus support ASK1 inhibition as a promising therapeutic target in heart failure. In particular, we propose that orally active kinase inhibitors with relative specificity for ASK1 might represent a means of augmenting ANP secretion, ANP plasma levels, and ANP activity in states of relative deficiency despite an increased level of ANP gene expression.

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REFERENCES

1. McGrath, M. F., de Bold, M. L., and de Bold, A. J. (2005) The endocrine function of the heart. *Trends Endocrinol. Metab. 16*, 469–477.
2. Cataliotti, A., and Burnett, J. C., Jr. (2005) Natriuretic peptides: Novel therapeutic targets in heart failure. *J. Invest. Med. 53*, 378–384.
3. Fukai, D., Wada, A., Tsutamoto, T., and Kinoshita, M. (1998) Short-term and long-term inhibition of endogenous atrial natriuretic peptide in dogs with early-stage heart failure. *Jpn. Circ. J. 62*, 604–610.
4. Yamamoto, K., Burnett, J. C., Jr., and Redfield, M. M. (1997) Effect of endogenous natriuretic peptide system on ventricular and coronary function in failing heart. *Am. J. Physiol. 273*, H2406–H2414.
5. Ito, T., Yoshimura, M., Nakamura, S., Nakayama, M., Shimasaki, Y., Harada, E., Mizuno, Y., Yamamuro, M., Harada, M., Saito, Y., Nakao, K., Kurihara, H., Yasue, H., and Ogawa, H. (2003) Inhibitory effect of natriuretic peptides on aldosterone synthase gene expression in cultured neonatal rat cardiocytes. *Circulation 107*, 807–810.
6. Lewicki, J. A., Greenberg, B., Yamanaka, M., Vlasuk, G., Brewer, M., Gardner, D., Baxter, J., Johnson, L. K., and Fiddes, J. C. (1986) Cloning, sequence analysis, and processing of the rat and human atrial natriuretic peptide precursors. *Fed. Proc. 45*, 2086–2090.
7. Rosenkranz, A. C., Woods, R. L., Dusting, G. J., and Ritchie, R. H. (2003) Antihypertrophic actions of the natriuretic peptides in adult rat cardiomyocytes: Importance of cyclic GMP. *Cardiovasc. Res. 57*, 515–522.
8. Kishimoto, I., Rossi, K., and Garbers, D. L. (2001) A genetic model provides evidence that the receptor for atrial natriuretic peptide (guanylyl cyclase-A) inhibits cardiac ventricular myocyte hypertrophy. *Proc. Natl. Acad. Sci. U.S.A. 98*, 2703–2706.
9. Yan, W., Wu, F., Morser, J., and Wu, Q. (2000) Corin, a transmembrane cardiac serine protease, acts as a pro-atrial natriuretic peptide-converting enzyme. *Proc. Natl. Acad. Sci. U.S.A. 97*, 8525–8529.
10. Wolf, K., Kurtz, A., Pfeifer, M., Hocherl, K., Riegger, G. A., and Kramer, B. K. (2001) Different regulation of left ventricular ANP, BNP and adrenomedullin mRNA in the two-kidney, one-clip model of renovascular hypertension. *Pfluegers Arch. 442*, 212–217.
11. Ruskoaho, H., Kinnunen, P., Taskinen, T., Vuolteenaho, O., Leppaluoto, J., and Takala, T. E. (1989) Regulation of ventricular atrial natriuretic peptide release in hypertrophied rat myocardium. Effects of exercise. *Circulation 80*, 390–400.
12. Wang, X. S., Diener, K., Jannuzzi, D., Trollinger, D., Tan, T. H., Lichenstein, H., Zukowski, M., and Yao, Z. (1996) Molecular cloning and characterization of a novel protein kinase with a

catalytic domain homologous to mitogen-activated protein kinase kinase kinase. *J. Biol. Chem. 271*, 31607–31611.
13. Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science 275*, 90–94.
14. Matsukawa, J., Matsuzawa, A., Takeda, K., and Ichijo, H. (2004) The ASK1-MAP kinase cascades in mammalian stress response. *J. Biochem. 136*, 261–265.
15. Matsuzawa, A., and Ichijo, H. (2008) Redox control of cell fate by MAP kinase: Physiological roles of ASK1-MAP kinase pathway in stress signaling. *Biochim. Biophys. Acta* XXX–XXX.
16. Tobiume, K., Matsuzawa, A., Takahashi, T., Nishitoh, H., Morita, K., Takeda, K., Minowa, O., Miyazono, K., Noda, T., and Ichijo, H. (2001) ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. *EMBO Rep. 2*, 222–228.
17. Yamaguchi, O., Higuchi, Y., Hirotani, S., Kashiwase, K., Nakayama, H., Hikoso, S., Takeda, T., Watanabe, T., Asahi, M., Taniike, M., Matsumura, Y., Tsujimoto, I., Hongo, K., Kusakari, Y., Kurihara, S., Nishida, K., Ichijo, H., Hori, M., and Otsu, K. (2003) Targeted deletion of apoptosis signal-regulating kinase 1 attenuates left ventricular remodeling. *Proc. Natl. Acad. Sci. U.S.A. 100*, 15883–15888.
18. Pickering, T. G. (2002) Effects of stress and behavioral interventions in hypertension: The rise and fall of omapatrilat. *J. Clin. Hypertens. (Darien, CT, U.S.) 4*, 371–373.
19. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Sideman, J., Smith, J., and Struhl, K. (1987) *Current Protocol in Molecular Biology*, John Wiley and Sons, New York.
20. He, X., Liu, Y., Sharma, V., Dirksen, R. T., Waugh, R., Sheu, S. S., and Min, W. (2003) ASK1 associates with troponin T and induces troponin T phosphorylation and contractile dysfunction in cardiomyocytes. *Am. J. Pathol. 163*, 243–251.
21. Liu, Y., Yin, G., Surapisitchat, J., Berk, B. C., and Min, W. (2001) Laminar flow inhibits TNF-induced ASK1 activation by preventing dissociation of ASK1 from its inhibitor 14-3-3. *J. Clin. Invest. 107*, 917–923.
22. Taniike, M., Yamaguchi, O., Tsujimoto, I., Hikoso, S., Takeda, T., Nakai, A., Omiya, S., Mizote, I., Nakano, Y., Higuchi, Y., Matsumura, Y., Nishida, K., Ichijo, H., Hori, M., and Otsu, K. (2008) Apoptosis signal-regulating kinase 1/p38 signaling pathway negatively regulates physiological hypertrophy. *Circulation 117*, 545–552.
23. Nishikimi, T., Maeda, N., and Matsuoka, H. (2006) The role of natriuretic peptides in cardioprotection. *Cardiovasc. Res. 69*, 318–328.
24. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J. 17*, 2596–2606.
25. Morita, K., Saitoh, M., Tobiume, K., Matsuura, H., Enomoto, S., Nishitoh, H., and Ichijo, H. (2001) Negative feedback regulation of ASK1 by protein phosphatase 5 (PP5) in response to oxidative stress. *EMBO J. 20*, 6028–6036.
26. Yuan, Z. Q., Feldman, R. I., Sussman, G. E., Coppola, D., Nicosia, S. V., and Cheng, J. Q. (2003) AKT2 inhibition of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1: Implication of AKT2 in chemoresistance. *J. Biol. Chem. 278*, 23432–23440.
27. Liu, Q., Wilkins, B. J., Lee, Y. J., Ichijo, H., and Molkentin, J. D. (2006) Direct interaction and reciprocal regulation between ASK1 and calcineurin-NFAT control cardiomyocyte death and growth. *Mol. Cell. Biol. 26*, 3785–3797.
28. Takeda, K., Shimozono, R., Noguchi, T., Umeda, T., Morimoto, Y., Naguro, I., Tobiume, K., Saitoh, M., Matsuzawa, A., and Ichijo, H. (2007) Apoptosis signal-regulating kinase (ASK) 2 functions as a mitogen-activated protein kinase kinase kinase in a heteromeric complex with ASK1. *J. Biol. Chem. 282*, 7522–7531.
29. Yasue, H., Obata, K., Okumura, K., Kurose, M., Ogawa, H., Matsuyama, K., Jougasaki, M., Saito, Y., Nakao, K., and Imura, H. (1989) Increased secretion of atrial natriuretic polypeptide from the left ventricle in patients with dilated cardiomyopathy. *J. Clin. Invest. 83*, 46–51.
30. Biondi, R. M., and Nebreda, A. R. (2003) Signalling specificity of Ser/Thr protein kinases through docking-site-mediated interactions. *Biochem. J. 372*, 1–13.
31. Giordano, F. J. (2005) Oxygen, oxidative stress, hypoxia, and heart failure. *J. Clin. Invest. 115*, 500–508.
32. Higuchi, Y., Otsu, K., Nishida, K., Hirotani, S., Nakayama, H., Yamaguchi, O., Hikoso, S., Kashiwase, K., Takeda, T., Watanabe, T., Mano, T., Matsumura, Y., Ueno, H., and Hori, M. (2003) The small GTP-binding protein Rac1 induces cardiac myocyte hypertrophy through the activation of apoptosis signal-regulating kinase 1 and nuclear factor-κ B. *J. Biol. Chem. 278*, 20770–20777.
33. Xiang, G., Seki, T., Schuster, M. D., Witkowski, P., Boyle, A. J., See, F., Martens, T. P., Kocher, A., Sondermeijer, H., Krum, H., and Itescu, S. (2005) Catalytic degradation of vitamin D up-regulated protein 1 mRNA enhances cardiomyocyte survival and prevents left ventricular remodeling after myocardial ischemia. *J. Biol. Chem. 280*, 39394–39402.
34. Kashiwase, K., Higuchi, Y., Hirotani, S., Yamaguchi, O., Hikoso, S., Takeda, T., Watanabe, T., Taniike, M., Nakai, A., Tsujimoto, I., Matsumura, Y., Ueno, H., Nishida, K., Hori, M., and Otsu, K. (2005) CaMKII activates ASK1 and NF-κB to induce cardiomyocyte hypertrophy. *Biochem. Biophys. Res. Commun. 327*, 136–142.
35. Szegezdi, E., Logue, S. E., Gorman, A. M., and Samali, A. (2006) Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep. 7*, 880–885.
36. Trombetta, E. S., and Parodi, A. J. (2003) Quality control and protein folding in the secretory pathway. *Annu. Rev. Cell Dev. Biol. 19*, 649–676.
37. Bloch, K. D., Jones, S. W., Preibisch, G., Seipke, G., Seidman, C. E., and Seidman, J. G. (1987) Proatrial natriuretic factor is phosphorylated by rat cardiocytes in culture. *J. Biol. Chem. 262*, 9956–9961.
38. DeWitt, N. D., dos Santos, C. F., Allen, K. E., and Slayman, C. W. (1998) Phosphorylation region of the yeast plasma-membrane H^+-ATPase. Role in protein folding and biogenesis. *J. Biol. Chem. 273*, 21744–21751.
39. Redondo, J., Bishop, J. E., and Wilkins, M. R. (1998) Effect of atrial natriuretic peptide and cyclic GMP phosphodiesterase inhibition on collagen synthesis by adult cardiac fibroblasts. *Br. J. Pharmacol. 124*, 1455–1462.
40. Feng, J. A., Perry, G., Mori, T., Hayashi, T., Oparil, S., and Chen, Y. F. (2003) Pressure-independent enhancement of cardiac hypertrophy in atrial natriuretic peptide-deficient mice. *Clin. Exp. Pharmacol. Physiol. 30*, 343–349.
41. Franco, V., Chen, Y. F., Oparil, S., Feng, J. A., Wang, D., Hage, F., and Perry, G. (2004) Atrial natriuretic peptide dose-dependently inhibits pressure overload-induced cardiac remodeling. *Hypertension 44*, 746–750.

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